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Article

Automated Sample Pretreatment Device Based on Microfluidic Chip Online Coupled with ICP-MS for Bacteremia Diagnosis

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ABSTRACT: Bacteremia is an extremely serious infectious disease with high mortality rate and increasing incidence. Early diagnosis and prompt treatment is key to improve the cure rate. In this study, we developed an automated sample pretreatment device based on microfluidic chip and online coupled it with ICP-MS for the simultaneous identification of E. Coli O157:H7 and Salmonella in human blood. This method realized automated sample loading, pathogen capture, elemental labeling, and signal desorption on the microfluidic chip through an automated liquid transfer system and E. Coli O157:H7 and Salmonella were detected simultaneously online by ICP-MS. The linear ranges of 400-80,000 CFU mL^{-1} for E. coli O157:H7 and Salmonella, as well as a method detection limits of 200 CFU mL^{-1} for E. Coli O157:H7 and 152 CFU mL^{-1} for Salmonella were obtained under optimum experimental conditions. The proposed approach realized the automated sample pretreatment and the simultaneous identification of two different kinds of bacteria in human whole blood, avoiding cumbersome sample pretreatment operations and sample contamination in conventional ICP-MS based procedures with elemental labeling strategy for bacteria analysis, demonstrating a strong potential for the clinical applications of bacteremia diagnosis.

INTRODUCTION

Bacteremia is a severe infectious disease that occurs when pathogenic bacteria grow and proliferate after invading the blood circulation system, spreading throughout the body.[1] When the immune system of the body is compromised, localized infections could disseminate to remote tissues and organs, resulting in the development of abscesses, peritonitis, endocarditis, and even meningitis. If the condition deteriorates, it may progress into sepsis accompanied by shock symptoms, ultimately culminating in mortality.[2] The bacterial resistance caused by the extensive application of broad-spectrum antibiotics and the prevalence of various invasive diagnostic and therapeutic procedures have increased the incidence of bacteremia yearly with a high rate of death. Early diagnosis and prompt treatment is key to improve the cure rate.[3]

Currently, the clinical diagnosis of bacteremia mainly relies on the culture of blood. Due to the extremely low concentration of bacteria in the blood, bacteria need to be proliferated by plate culture prior to identification and investigation, which takes 5-7 days, significantly delaying diagnosis and treatment[3]. Additionally, the process requires multiple blood collections and the consumption of blood samples is at the milliliter level, causing patient discomfort and poor compliance. In recent years, immunological and molecular biological detection techniques such as enzyme-linked immunosorbent assay,[4] immunochromatography[5], immunofluorescence,[6] polymerase isothermal amplification,[7] electrochemical analysis,[8] and surface-enhanced Raman scattering[9] have developed rapidly, greatly improving detection sensitivity of bacteria analysis and reducing sample consumption. However, due to the diversity of pathogens and the strict environmental requirements for bacteria analysis, these methods still have some limitations: (1) The diversity of environmental microorganisms makes manual sample pre- treatment

Fig. 1 The principle of this work.

treatment prone to contamination, resulting in false positives; (2) Most methods can only detect specific target bacteria and cannot screen for multiple bacteria simultaneously, making it difficult to identify the type of bacterial infection quickly. Therefore, it is urgent to develop new diagnostic methods for bacteremia.

Elemental labeling approach with inductively coupled plasma mass spectrometry (ICP-MS) is a novel bioquantitative analysis method developing rapidly in the past several years, which has been widely applied to the quantitative analysis of nucleic acids,[10] proteins,[11] cells,[12,13] viruses,[14] and pathogens.[15] In addition, ICP-MS with elemental labeling approach combined with microfluidic chip has realized the single cell detection.[16,17] This technique holds promise for overcoming the bottlenecks in current bacteremia diagnostic techniques due to its good compatibility with a variety of sample pretreatment methods and unique advantages in multi-target detection.[18-19] However, the direct application of element-tagged ICP-MS methods in the bacteremia diagnostic still faces challenges. Currently, the application of ICP-MS technique with elemental labeling approach in bacteria analysis can be categorized into two main approaches: one based on pathogen surface markers[20-22] and the other on characteristic nucleic acid sequences.[23-25] The former approach needs multiple procedures, such as bacteria capture, elemental labeling, signal desorption. The latter approach requires the extraction of nucleic acids from bacteria in blood samples before their quantitative analysis. Both methods demand complicated pre-treatment steps that are performed manually, which can easily lead to blood sample contamination and false-positive results. Therefore, to fully develop the potential of ICP-MS technique with elemental labeling approach in the diagnosis of bacteremia, the development of automated sample pre-treatment devices is urgently needed.

Microfluidic chip technology based on microfabrication technology, is characterized by microchannel networks that control fluids at the micron scale. It integrates sampling, reacting, diluting, reagent adding, separating, and detecting on a chip with a few square centimeters in size, realizing various functions of a conventional chemical or biological laboratory.[26] Microfluidic chip has the advantages of integration, high throughput, and ease of automation, avoiding contamination risks and personal error resulted from cumbersome manual operations and achieving high-throughput automated analysis.[27] Therefore, developing automated sample pretreatment device based on microfluidic chip can solve the problem of easy contamination of sample in the current ICP-MS based techniques for pathogen identification, avoiding the risks of false positives.

This work aims to develop an automated sample pretreatment device based on microfluidic chip online coupled with ICP-MS using an elemental labeling approach to identify two kinds of bacteria, addressing the cumbersome manual pretreatment operations and contamination issues in the current ICP-MS based techniques for bacteria identification and quantification. The conceptual approach is illustrated in Fig.1A: By constructing a serpentine reaction channel on a microfluidic chip and manipulating biological magnetic beads through an external magnetic field, we developed an immunoassay for E. Coli O157:H7 and Salmonella on a microfluidic chip coupled with ICP-MS with elemental labeling approach. As shown in Fig.1B, a gas-driven automated liquid transfer system consisted of a driving gas supply module and a gas control module was designed for automated pretreatment operations such as bacteria capture, elemental labeling, and signal desorption. The automated pretreatment operations were completed by the specific settings of a PLC controller (programmable logic controller) in the gas control module, which operates downstream solenoid valves according to preset programs, controlling the pressure in the sample and reagent bottles to programmatically introduce samples and various reagents, avoiding the cumbersome operations and contamination of traditional bacteria immunoassay based on ICP-MS with elemental labeling approach. Finally, online ICP-MS detection realized simultaneous analysis of E. Coli O157:H7 and Salmonella in human blood.

EXPERIMENTAL

Materials and instruments. Detailed information was displayed in Supporting Information. The operating parameters of ICP-MS are listed in Table S1.

Design and preparation of the microfluidic chip. The design and physical image of the microfluidic chip are shown in Fig. 2(a) and (b). The chip consists of a serpentine channel, 4 inlets (A1-A4), waste outlet (A5), and outlet of online connection port for ICP-MS (A6). A1-A4 are sample inlet, washing buffer inlet, elemental tag inlet and elution inlet, respectively. Magnetic beads on the chip are fixed by high-intensity neodymium-iron-boron permanent magnets (2.5×0.6×0.3 cm). The channel height is 200 μm, width is 300 μm, and length is 846 μm. Fig. 2(c) shows a 10x magnified image of the channel filled with magnetic beads under a microscope, indicating uniform and dense filling.

Design and fabrication of the automated liquid transfer system. The schematic and physical images of the automated liquid transfer system are shown in Fig. 3(a) and (b). The system consists of a driving gas supply module and a gas control module. The driving gas supply module includes a micro air pump and an oil-water separator. The gas control module, the core of the automated sampling system, includes a PLC controller, an array of solenoid valves, and a manifold. The PLC controller in the gas control module operates downstream solenoid valves according to the preset programs, controlling the pressure in the sample and reagent bottles to programmatically introduce samples and various reagents.

Online design of the microfluidic chip and ICP-MS. The chip's A6 outlet is connected to the ICP-MS PTFE sample tube (inner diameter 0.44 mm, length 15 cm) via a PTFE tube (inner diameter 2 mm, length 3 cm). The sample tube was connected to a micro concentric nebulizer with a nebulizer flow rate of 0.3 mL min^{-1} and a microfluidic chip flow rate of 5 μL s^{-1}, ensuring flow rates matching for online coupling.

Bacterial culture. A little bacterial suspension from glycerol stock tubes of E. Coli O157:H7 and Salmonella was picked using a 1μL sterile inoculating loop. The bacteria were steaked three times on a solid TSA medium and incubated at 37 ºC for 24 h. A single bacterial colony from the solid medium was then exchanged to TSB fluid medium and incubated for 16-18 h at 37°C on a shaker in 170 rpm to produce that high-concentration suspension of bacteria. At this stage, the bacterial suspension's OD600 was around 1.0, meaning there were 10^8 CFU mL^{-1} of bacteria present. Subsequently, 1 mL of each suspension of E. Coli O157:H7 and salmonella was transferred into 1.5 mL sterile EP tubes, then centrifuged for one min at 12000 rpm. discarding the supernatant, and the resulting pellet of bacteria was used for the next step of the experiment.

Fig. 2 (a) the design drawing of the microfluidic chip; (b) the physical image of the designed microfluidic chip; (c) 10x magnified image of the channel filled with magnetic nanoparticles under a microscope.

Fig. 3 (a) The schematic images; (b) physical images of the automated liquid transfer system.

Synthesis of capturing probe of MBs-anti-7C2 and MBs-anti-8G3. After washed with 0.01 mol L^{-1} NaOH and ultrapure water, 100 μL of 10 mg mL^{-1} MBs-COOH were activated with 20 mg mL^{-1} EDC in 200 μL at 30 min. Subsequently, anti-8G3 or anti-7C2 (2 μg), 90 μL of 100 mmol L^{-1} MES at pH 5.5 and 6 μL of 100 mmol L^{-1} MEST were added. Following a 4-hour reaction, the MBs underwent magnetic separation and three rounds of 0.1% PBST washing to remove extra antibodies. MBs-anti-7C2 and MBs-anti-8G3, the resultant products, were then dissolved in 100 μL of PBS in store at 4 °C.

Synthesis of labeling probes of Ag NP-anti-8B1 and Au NP-anti-5H12. 100 μL with Au NP solution (100 mg mL^{-1}; K_2CO_3 set to pH 9.0) was mixed with 3 μg of anti-5H12, for an overnight reaction at 4°C, then 20 μL of 5% BSA was used to block the unreacted sites of the Au NPs. The products were centrifuged at 12000 rpm for 5 min, with the supernatant discarded to remove excess anti-5H12. The Au NP-anti-5H12 were washed with PBS for three times, finally dispersed in 100 μL of PBS. The steps for Ag NP-anti-8B1 are similar.

Immunoassay on microfluidic chip and ICP-MS detection. 100 μL of 5 mg L^{-1} capturing probe of MBs-anti-7C2 and MBs-anti-8G3 are injected into the chip channel using a 200 μL pipette and magnetically fixed for 5 min. Subsequently, 100 μL of sample

is flowed through the serpentine channel from the A1 inlet at 5 μL s^{-1}. Once the channel is filled, a reaction time of 20 min is allowed for sufficient capture. Following this, 100 μL of PBS buffer is flowed through the A2 inlet at 5 μL s^{-1} to wash away complex sample matrix, and the washing liquid is discharged through the A5 waste outlet. After a 3-minute wash, 100 μL of labeling probes of AgNP-anti-8B1 and AuNP-anti-5H12 are flowed through the A3 inlet at 5 μL s^{-1} into the serpentine channel, and the system is allowed to stand for 20 min for sufficient labeling. A subsequent wash with 100 μL of PBS buffer through the A2 inlet at 5 μL s^{-1} is performed to remove excess labeling probes, with the washing liquid discharged through the A5 waste outlet. After a 3-minute wash, 100 μL of 1.2 mol L^{-1} formic acid is flowed through the A4 inlet into the serpentine channel and allowed to stand for 15 min. The outflow is directly introduced into the ICP-MS concentric nebulizer through the A6 online connection interface. The introduction and discharge of liquids are completed by an automated liquid transfer system, with the specific settings of the PLC in Table S2.

Real sample analysis. In compliance with the guidelines of the regional ethics council, healthy human blood samples (2 mL, purple lid EDTA-K2 vacuum containers) from individuals were taken by Public Health Clinical Center of Chengdu in order to conduct spiking recovery studies. Each 1 mL of sample was spiked with 10 μL of 500, 2000 and 10000 CFU of E. Coli O157: H7 as well as Salmonella. Then, the red blood cell lysis buffer was used to lyse the red blood cells in samples to reduce the sample matrix interference, and the centrifugation was run for three min at 12,000 rpm. Following the supernatant's disposal, the centrifugal products were washed by PBS for three times and resuspended in 100 μL of PBS, then proceeded with subsequent microfluidic chip immunoassay and ICP-MS analysis.

RESULTS AND DISCUSSION

Optimization of the parameters of automated sample pretreatment device

(1) Optimization of microfluidic chip channel width. The channel width of the microfluidic chip determines the amount of capture probes that can be immobilized, which in turn affects the capture efficiency of pathogens, directly influencing the detection sensitivity. Therefore, the channel width of the microfluidic chip was optimized. The ^{197}Au and ^{107}Ag signal intensity of the immunoassay on microfluidic chip was examined by ICP-MS at channel widths ranging from 50 to 500 μm as shown in Fig. S1. It was found that from 50 to 200 μm, as the channel width increased, the immobilization area of the magnetic beads increased, and the capture efficiency also increased, resulting in an increasing signal. When the width reached 200 μm, the signal gradually stabilized. Finally, 300 μm was chosen, and at this width, the amount of magnetic beads immobilized on the microfluidic chip was 1 mg.

(2) Optimization of flow rate for automated liquid transfer system. To achieve online coupling of the automated liquid transfer system with ICP-MS, the flow rate of the automated liquid transfer system was optimized. The concentric nebulizer equipped with ICP-MS can achieve a rate of 0.1-1 mL per minute (1.7-17 μL s^{-1}), while the pressure range of the automated liquid transfer system can be adjusted from 0.00 to 0.08 MPa, corresponding to a range of 0 to 50 μL s^{-1} for flow rate. More than 50 μL s^{-1} of flow could result in poor flow stability. Therefore, the flow rate for online coupling of the automated liquid transfer system with ICP-MS was optimized within the range of 2-16 μL s^{-1}. As observed in Fig. S2, the intensity of ^{197}Au and ^{107}Ag signal steadily rose as the flow rate increased from 2 to 4 μL s^{-1}. Within the range from 4 to 8 μL s^{-1}, the signal steadily became stable. When the flow rate increased further, ^{197}Au and ^{107}Ag gradually decreased due to reduced nebulization efficiency. Considering that a high flow rate might affect the stability of magnetic bead immobilization, 5 μL s^{-1} and 0.01 MPa were chosen as the final flow rate and pressure for the automated liquid transfer system.

(3) Characterization of capturing and labeling probes. UV spectroscopy was used to characterize the successful preparation of MBs-anti-8G3 and MBs-anti-7C2, depending on the UV typical peak of absorption of proteins approximately 280 nm. A notable decrease in UV absorption peak of the anti-8G3 and anti-7C2 residue at 280 nm was observed, indicating that the antibodies and MBs-COOH have successfully coupled. And the maximum UV absorption peak of Ag NP and Au NP had a red shift after binding with antibodies, indicating successful reaction. Meanwhile, the particle sizes of Ag NP and Au NP were figured out to be approximately 20 nm by TEM characterization. The detailed data for characterization of capturing and labeling probes were shown in our previous work.[28]

Investigation of immunoreaction conditions

***(1)* Effect of capture time.** The variations in ^{197}Au and ^{107}Ag signal intensities were examined when the capture time of magnetic beads for E. coli O157: H7 and Salmonella varying from 10 to 80 min. As illustrated in Fig. S3, it indicated throughout a 20-min period, the signal of ^{197}Au and ^{107}Ag steadily grew as the incubation time increased; when the incubation time continued to increase, the ^{197}Au and ^{107}Ag slightly decreased. Finally, 20 min was used as the capture time in the following experiments.

(2) Effect of labeling Time. Labeling time of Ag NP-anti-8B1 and Au NP-anti-5H12 for E. Coli O157: H7 and Salmonella were optimized. A labeling time from 10 to 80 min was investigated on the signal intensity of ^{107}Ag and ^{197}Au, as shown in Fig. S4. The signal of ^{107}Ag and ^{197}Au steadily rose when the labeling time

increased from 10 to 20 min. After 20 min, the signal intensity of ^{107}Ag and ^{197}Au reached a platform. Finally, 20 min was chosen for the following experiments.

(3) Effect of desorption conditions. Acid as a desorption agent was employed in this work to break down the antigen-antibody association, desorbing ^{107}Ag and ^{197}Au signal from the immune system for ICP-MS analysis. First, the type of desorption agent was optimized. 1.0 mol L^{-1} of citric acid, nitric acid, formic acid, and acetic acid were investigated as desorption agent respectively. As shown in Fig. S5(a), when 1.0 mol L^{-1} nitric acid was used as the elution agent, The extremely low signal of ^{107}Ag and ^{197}Au was observed possibly due to the aggregation of Au NP and Ag NP in a strong acid medium, which would result in undetectable signals. Compared with citric acid and acetic acid, formic acid resulted in the highest signal for ^{107}Ag and ^{197}Au, and was finally selected to serve for the desorption procedure. Next, the effect of desorption agent concentration ranging from 0.1 to 2.0 mol L^{-1} on the signal intensity of ^{107}Ag and ^{197}Au was investigated. As shown in Fig. S5(b), the intensity of the ^{107}Ag and ^{197}Au signals progressively rose as the concentration of formic acid increased, reaching a plateau at 1.0 mol L^{-1}. Finally, it was determined that the optimal formic acid concentration was 1.2 mol L^{-1}. Continuous desorption with 100 μL of 1.2 mol L^{-1} of formic acid was performed three times for the optimization of the volume of the desorption agent to confirm whether the first desorption was complete. As shown in Fig. S5(c), 100 μL of 1.2 mol L^{-1} of formic acid could achieve complete elution of ^{107}Ag and ^{197}Au. Therefore, 100 μL was selected as the final desorption volume. The influence of desorption time on the signal intensity of ^{107}Ag and ^{197}Au was examined at 5, 10, 15, 20, 30, and 40 min. As revealed in Fig. S5(d), the intensity of ^{107}Ag and ^{197}Au signal rose with desorption time range of 5 to 10 min, reaching a plateau after 15 min. Therefore, the final desorption time was set at 15 min.

Investigation of chip usage life. Experiments were carried out to investigate the times that the chip could be reused. After each immunoassay, the chip was washed with PBS for 5 min by the automated sample pretreatment device and reassembled with magnetic beads, and then went through the next immunoassay, the ICP-MS was used to record the signal intensity of ^{107}Ag and ^{197}Au for each immunoassay. As shown in Fig. S6, it was found that the chip could be used more than 50 times.

Analytical performance

Evaluation of this method's analytical performance was conducted under optimal experimental conditions. The concentration of Salmonella and E. Coli O157: H7 of 400, 1000, 2000, 4000, 10000, 20000, 40000, 80000 CFU mL^{-1} was used to investigate the linearity of the proposed method. As shown in Fig. 4, the linear range for detecting Salmonella and E. Coli O157: H7 was 400-80000 CFU mL^{-1}. According to the IUPAC, the LOD was defined as 3σ/k, in which σ means the standard deviation of blank experiment and k means the slope of the calibration curve, the LOD of 200 CFU mL^{-1} for E. coli O157: H7 and 152 CFU mL^{-1} for Salmonella were obtained. The RSD (n=7, cell number=1000) of the proposed method for the detection of E. coli O157: H7 and Salmonella were 7.3% and 8.2%, respectively, demonstrating good stability and reproducibility of the proposed method. This method's analytical performance was compared with that of other approaches[29-34] reported in the literature shown in Table S3. The sensitivity of this proposed method is at a moderate level. However, this method achieves automated sample loading, bacteria capture, elemental labeling, and signal desorption through an automated sample pre-treatment device based on a microfluidic chip. And it realized the automated sample pre-treatment and simultaneous quantification of two kinds of bacteria by ICP-MS and ultimately applied to the analysis of human whole blood samples, showing potential for clinical applications.

Fig. 4 Analytical performance of the proposed method for Salmonella (a) and E. coli O157: H7 (b). AgNP-anti-8B1 and AuNP-anti-5H12, 10 μL; the amount of MBs-anti-7C2 and MBs-anti-8G3, 1 mg. Error bar represents s.d. value for triplicate analysis.

Fig. 5 The specificity of the method. AgNP-anti-8B1 and AuNP-anti-5H12, 10 μL; Salmonella and E. Coli O157: H7, Listeria monocytogenes, and Staphylococcus aureus, 2.0×10^4; the amount of MBs-anti-7C2 and MBs-anti-8G3, 1 mg. Error bar represents s.d. value for triplicate analysis.

Method Specificity. The specificity of the method was assessed. MBs-anti-8G3 and MBs-anti-7C2 were used to capture E. coli O157: H7, Salmonella, Listeria monocytogenes, and Staphylococcus

Table 1. The results of spiking experiments in normal human peripheral blood sample

Sample	Salmonella					E. coli O157: H7				
	Added ($\times10^2$CFU mL^{-1})	This method		Blood culture method		Added ($\times10^2$CFU mL^{-1})	This method		Blood culture method	
		Found ($\times10^2$ CFU mL^{-1})	Recovery (%)	Found ($\times10^2$ CFU mL^{-1})	Recovery (%)		Found ($\times10^2$ CFU mL^{-1})	Recovery (%)	Found ($\times10^2$ CFU mL^{-1})	Recovery (%)
1	5.00	4.94±0.12	98.8	4.25±0.10	85.0	5.00	5.15±0.30	103	5.18±0.40	104
	20.0	18.7±1.4	93.5	20.2±1.8	101	20.0	19.2±1.5	96.0	19.5±1.8	97.5
	100	104±9.2	104	110±9.9	110	100	87.3±5.7	87.3	112±5.3	112
2	5.00	4.85±0.20	97.0	4.92±0.20	98.4	5.00	5.21±0.50	104	4.79±0.51	95.8
	20.0	19.2±1.2	96.0	19.5±2.0	97.5	20.0	19.1±1.3	95.5	19.2±1.4	96.0
	100	101±9.4	101	92.0±7.0	92.0	100	103±7.2	103	108±7.0	102
3	5.00	4.34±0.30	86.8	5.40±0.20	108	5.00	4.92±0.30	98.4	4.74±0.52	94.8
	20.0	19.3±1.8	96.5	20.8±2.1	104	20.0	18.9±1.4	94.5	18.0±1.6	90.0
	100	106.0±4.8	106	92.0±9.1	92.0	100	104±6.2	104	97.0±5.2	97.0

aureus respectively. Ag NP-anti-8B1 and Au NP-anti-5H12 were used as probes to label E. coli O157: H7, Salmonella, Listeria monocytogenes, and Staphylococcus aureus. The signals of ^{107}Ag and ^{197}Au were simultaneously detected using ICP-MS, as shown in Fig. 5. It can be seen that for Salmonella, a significant signal response was only observed when the labeling probe was Au NP-anti-5H12; for E. coli O157: H7, an obvious signal was obtained only when the labeling probe was Ag NP-anti-8B1. No discernible signal was observed for Listeria monocytogenes or Staphylococcus aureus, irrespective of the presence of Ag NP-anti-8B1 or Au NP-anti-5H12. This indicates that the method demonstrates a good specificity in identifying and quantification of E. Coli O157: H7 and Salmonella.

Recovery. Spiking experiments were carried out for the investigation of recovery on three healthy human peripheral blood samples, and the results were shown in Table 1. It can be shown that this proposed method has good recoveries ranging from 86.8 to 106%. Meanwhile, the experimental results obtained by this method matched those obtained by the blood culture method in clinical use, indicating a good potential of the proposed method for clinical applications.

CONCLUSION

This work established an automated sample pre-treatment device based on a microfluidic chip, which could be online coupled with ICP-MS to identify and quantify two kinds of bacteria. It realized the automated capture, elemental labeling, signal desorption and simultaneous ICP-MS detection of E. Coli O157:H7 and Salmonella in human blood, avoiding the problems of cumbersome sample pre-processing operations and sample contamination in conventional ICP-MS based method for bacteria detection. Moreover, this method is suitable for the analysis of human whole blood samples and shows potential for clinical applications, providing a new approach for the diagnosis of bacteremia.

AUTHOR INFORMATION

Luelue Huang received her PhD degree in Processing and Storage of Agriculture from Jiangnan University in 2011. Now, she is a professor at Food and Drug school of Shenzhen Polytechnic University. She focuses on the development of mass spectrometry and terahertz technology in food safety and quality control. She is author or co-author of over 30 articles published in peer-reviewed scientific journals.

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Notes

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